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Establishment of cell lines stably expressing dCas-fusions to address kinetics of epigenetic editing

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Running Header – kinetics of epigenetic editing

Abstract

Epigenetic editing is a promising approach to modulate the local chromatin environment of target genes with the ultimate goal of stable gene expression reprogramming. Epigenetic editing tools minimally consist of a DNA binding domain and an effector domain. The CRISPR-dCas9 platform, where mutations in the nuclease domains render the Cas9 protein inactive, is widely used to guide epigenetic effectors to their intended genomic loci. Its flexible nature, simple use and relatively low cost have revolutionised the research field of epigenetic editing. Although effective expression modulation is readily achieved, only a few studies have addressed the maintenance of the induced effects on endogenous loci. Here, we describe a detailed protocol to engineer cells that stably express the CRISPR-dCas9-effectors. The protocol involves modification of published dCas9-based plasmid vectors for easy transfer of the effector domain between the vector designed for transient transfection and the vector used for establishing cell lines stably expressing the dCas9-effector fusion protein. Transient transfection of the dCas9-effector producing cells with sgRNA expressing plasmids allows studying of the maintenance of epigenetic editing. Targeting various genes in different

chromatin contexts and/or co-targeting multiple CRISPR-dCas9-effectors can be used to unravel rules underlying maintained gene expression reprogramming.

Key Words: CRISPR-dCas9; fusion proteins, epigenetic reprogramming; epigenetic memory

1 Introduction

Epigenetic editing has the potential to treat or even cure diseases by stably reprogramming the chromatin environment and achieving maintained gene activation or gene silencing. To this end, a one-and-done approach is envisioned, where patients would receive treatments with epigenetic editing tools, after which the rewritten epigenetic environment is stably maintained. In general, DNA methylation and histone modifications are faithfully copied during cell divisions to assure maintenance of the cell phenotype [1-3]. Therefore, deliberately editing epigenetic marks bears the promise to make permanent changes [4-7]. However, conflicting data exist with regard to the maintenance of the newly established epigenetic states. For example, gene repression by DNA methylation has been shown to be maintained for the *SOX2* gene [6], while DNA methylation-induced repression was not maintained for the *VEGF-A* gene [8]. In this respect, Amabile *et al.* showed that for most loci efficient long-term memory of an induced repressive epigenetic state was only obtained after targeting a combination of repressors (KRAB-DNMT3A-DNMTL) [9].

Although various DNA binding platforms have been explored previously to target endogenous genes [10], none of them is as suitable as the CRISPR-dCas9 platform is for large scale multiplex investigations of the maintenance of epigenetic marks on gene expression [11]. An experimental system employing transient expression of sgRNAs in engineered cell lines stably

expressing dCas9-effector fusions thus would provide a flexible model to address the influence of native chromatin states on long-term kinetics of epigenetic editing. We previously used such a model to investigate the sustainability of *SPDEF* gene repression in MCF-7 cell lines stably expressing dCas9 fused either with the non-catalytic transcriptional repressor SKD, or with epigenetic editors and their mutants, G9a and SUV39h1 (for H3K9me), the SET domain of EZH2 (for H3K27me), or a chimeric DNMT3A-3L fusion (for DNA methylation). The sgRNAs targeting the *SPDEF* promoter region were introduced by transient transfection. Repression of gene expression was observed in cells expressing dCas9-SKD, -SUV39H1, -EZH2 and -DNMT3A-3L, but this repression was not maintained when measured after 14 days. Interestingly, the repression of *SPDEF* was sustained in cells expressing dCas9-G9a, while short-term expression of the sgRNAs in the dCas9-G9A mutant cells regained activation after 14 days [7].

Conceptually, sustained gene re-expression is more challenging to achieve than sustained silencing. Nevertheless, by inducing the endogenous expression of *Brn2*, *Ascl1*, and *Myt1l* genes, Black *et al.* were able to stably convert fibroblasts into neuronal cells. To this end, primary mouse embryonic fibroblasts were engineered to constitutively express VP64-dCas9-VP64, and sgRNAs were delivered through transient transfection to investigate the maintenance of the induced effects on the three neurogenic genes. Upon transiently targeting of VP64, sustained activation of the target genes was demonstrated at day 18, which was associated with effective reprogramming of these embryonic cells [12]. Although constitutive lentiviral expression induced neuronal reprogramming more effectively, these, and similar data for e.g. *MyoD1* [13, 14], indicate promise for transient, non-integrative molecular tools in stable cell reprogramming.

Despite these promising transdifferentiation data, we and others demonstrated that the induction of gene expression by non-catalytic VP64-fusions generally is transient in adult cells [15, 16]. The direct rewriting of chromatin signature states might be more effective in inducing sustained gene re-expression. Using doxycycline-inducible stable cells lines, we previously demonstrated for a hypermethylated gene, that although induction of local methylation of H3K4 is sufficient to directly initiate gene expression, no sustained gene re-expression could be observed [4]. Interestingly, the induced reactivation was sustained for a hypomethylated gene and even reinforced ten days after transfection [4].

To further identify factors that determine maintenance of epigenetic profiles, studies focusing on long-term kinetics of induced epigenetic changes are needed. Towards this end, stable cell lines can be engineered to continuously expressing dCas9-effectors, which can be transiently transfected with sgRNAs temporarily targeting the effectors to the genes of interest [7]. Although these cell lines constantly express either the transcriptional repressor (SKD), activator (VP64), or epigenetic editors, the sgRNAs can only for short time periods guide the effectors to the gene of interest (one-and-done approach). As controls, stable cell lines expressing mutant forms of the epigenetic editors will determine whether the observed effects can be attributed to the catalytic activity of the enzymes, or to for example steric hindrance or recruitment of other chromatin remodelers. Similarly, a stable cell line expressing dCas9 without an effector will control for the effects of dCas9 binding. Here, we provide detailed protocols to construct transient expression plasmids and stable dCas9 expressing cell lines and describe the procedure to transiently transfect these stable cells with sgRNA plasmids to investigate the kinetics of epigenetic editing.

2 Materials

2.1 Molecular biology reagents and equipment

1. Nuclease-free water
2. Plasmid DNA extraction kit
3. Restriction enzymes: AscI, AgeI, PacI, BsmBI, PacI, BamHI, NotI, MluI, AsiSI. For faster digestion the use of FastDigest preparations of Thermo Scientific or Timer-Saver qualified preparations of New England Biolabs is recommended.
4. T4 DNA ligase (400U/µl) and ligase buffer
5. High fidelity DNA polymerase (Phusion DNA polymerase) and buffer
6. dNTPs (10mM)
7. Oligonucleotides
   A. Sequencing primer 1: TACAAGGATGACGATGACAAG (pMLM3705 positions 4591 – 4612)
   B. Sequencing primer 2: TAGAAGGCACAGTCGAGG (pMLM3705 positions 4840 – 4857, complementary strand, BGH Reverse, https://www.addgene.org/mol-bio-reference/sequencing-primers/)
   C. Sequencing primer 3: CAGGGTTATTGTCTCATGAGCGG (MLM3636 positions 2183 – 2205, OS280 primer, https://www.addgene.org/43860/)
   D. Sequencing primer 4: CAGCGGGGCTGCTAAAGCGCATGC (pHAGE EF1α dCas9-VP64 positions 161-184, complementary strand, MSCV-rev primer)
   E. Oligo 1: CGCGCCCATATGTTAATTAACAATTAA (complementary to Oligo 2, the duplex has AscI- and AgeI-compatible single-stranded ends)

F. Oligo 2: CCGGTTAATTGTTAATTAACATATGGG (complementary to Oligo 1, the duplex has AscI- and AgeI-compatible single-stranded ends)

G. Oligo 3: GATCCACACGCGTTAAGCGATCGCGC (complementary to Oligo 4, the duplex has BamHI- and NotI-compatible single-stranded ends)

H. Oligo 4: GGCCGCGCGATCGCTTAACGCGTGTG (complementary to Oligo 3, the duplex has BamHI- and NotI-compatible single-stranded ends)

I. Primers for PCR amplification of effector domains: see below at the specific steps

8. Equipment for gel electrophoresis of DNA fragments
9. A thermocycler for PCR-amplification of DNA fragments
10. Agarose and Tris-borate-EDTA buffer (50 mM Tris base, 50 mM boric acid, 1 mM EDTA, pH8.3) for agarose gel electrophoresis
11. Ethidium bromide or other fluorescent dye for visualizing DNA fragments
12. DNA fragment size marker (e.g. 1 Kb Plus DNA Ladder)
13. Razor blades and gel extraction kit
14. *E. coli* (e.g. DH10B, DH5α) cloning host (chemically competent)
15. LB growth medium for bacteria
16. 100 mg/ml ampicillin stock solution in water, filter-sterilized
17. LB agar plates
18. 1 M Tris-HCl, pH7.5
19. 1 M NaCl
20. TE buffer: 10 mM Tis-HCl, pH 7.5
21. Nanodrop or similar equipment for measuring DNA concentration

2.2 Plasmids

1. pMLM3705 (A gift from Keith Joung, Addgene plasmid #47754)
2. MLM3636 (A gift from Keith Joung, Addgene plasmid #43860)
3. pHAGE EF1α dCas9-VP64 (A gift from Rene Maehr and Scot Wolfe, Addgene plasmid #50918)
4. psPAX2 (Lentiviral packaging plasmid; a gift from Didier Trono, Addgene plasmid #12260)
5. pMD2.G VSV-G (Envelope expressing plasmid; a gift from Didier Trono, Addgene plasmid #12259)

2.3 Cell culture and transfection (*see* **Notes 1** and **2**)

1. HEK293T cells (ATCC: CRL-3216) + Medium (DMEM + 10% FBS + 1% L-glutamine + 1% gentamicin)
2. Cell culture flasks and plates
3. Cell culture reagents (including Phosphate Buffered Saline, trypsin)
4. Incubator set to 37°C and 5% CO_2, humidified atmosphere
5. Transfection medium (f.e. Lipofectamine (LTX*PLUS), Plus reagent)
6. Host cells for transfection/transduction and suitable growth medium
7. 10 cm plates (tissue culture treated)
8. Polybrene (Hexadimethrine bromide)
9. Puromycin (1µg/ml)
10. 10 ml syringes
11. 0.45 µ Surfactant-Free Cellulose Acetate (SCFA) filter
12. 15 ml tubes

13. Disinfectant (f.e. 1% sodium hypochlorite) and an appropriate waste container (f.e. vacuum bag)

3 Methods

3.1 Construction of plasmids for transient expression of dCas9-effectors

To validate the effector function of dCas9-effector fusions before constructing the stable cell lines, the dCas9-effector and the sgRNA(s) can be tested in a transient expression system. To facilitate construction of fusions between dCas9 and different effectors, the plasmid pMLM3705 encoding a dCas9-VP64 fusion (Figure 1) is converted into a general acceptor plasmid by deleting the 159 bp AscI-AgeI fragment carrying the VP64 gene and replacing it with the double-stranded oligonucleotide Oligo 1 + Oligo 2 (see 2.1 step 7 E & F) containing a PacI site. Ligation of the Oligo 1 + Oligo 2 duplex to the pMLM3705 backbone restores the AscI site. The new plasmid called pdCas9-NED (No Effector Domain) will encode the fusion protein dCas9-SV40 nuclear localization signal (NLS) - 3xFLAG peptide, and will serve as an acceptor for genes of effector domains (Figure 1). PCR-amplified fragments coding for an effector can be cloned between the AscI and the PacI sites of pdCas9-NED (Figure 1).

3.1.1 Construction of the vector plasmid pdCas9-NED

1. Digestion of pMLM3705. Prepare the reaction mixture:
    - 2 to 4 µg pMLM3705 plasmid DNA
    - 10 µl 10X FastDigest Green Buffer
    - 2 µl FastDigest AscI
    - 2 µl FastDigest AgeI
    - H_2O to 100 µl final volume

Incubate for 1 hour at 37 °C, then check completeness of digestion by running 10 µl of the digestion mixture in a 1% agarose gel alongside with undigested control.

2. Annealing of Oligo 1 and Oligo 2. The duplex will contain a PacI site and carry AscI- and AgeI-compatible single-stranded ends.

   Dissolve the oligonucleotides to 100 µM in TE buffer. Combine:

   - 5µl Oligo 1
   - 5 µl Oligo 2
   - 1 µl 1 M Tris-HCl pH7.5
   - 2 µl 1 M NaCl
   - 7 µl H_2O

   Place the tube to 95 °C (a water bath or a thermocycler) for 5 minutes, then let it slowly cool down to room temperature. The annealed duplex can be stored in ice or at -20 °C.

3. If the digestion of pMLM3705 is complete (see 3.1.1. step 1), load the rest of the digestion mixture on a preparative 0.8% agarose gel, and isolate the large fragment (7626 kb) by using a gel extraction kit. Determine the concentration of the purified fragment by measuring A_{260} (Nanodrop).

4. Ligation of the annealed oligonucleotides to the purified vector fragment. Prepare the following reaction mixture:

   - ~200 ng linear vector DNA
   - Annealed Oligos 1+2 (2 to 10-fold molar excess over the vector)
   - 2 µl 10x T4 DNA ligase buffer
   - 1 µl T4 DNA ligase
   - H_2O to 20 µl final volume

Mix gently and incubate for 30 minutes - 2 hours at room temperature (*see* **Notes 3** and **4**).

5. Transformation of *E. coli* with the ligated DNA:
    - Add 2 µl ligation mixture to 100 µl competent *E. coli* cells
    - Mix gently
    - Place the tubes on ice for 30 minutes
    - Place the tubes to 42 °C for 2 minutes
    - After the heat-shock, pipette the mixture into a 15 ml sterile tube containing 500 µl LB medium.
    - Incubate for an hour in a rotating incubator at 37 °C
    - Spread 100 µl cell suspension on LB agar plates containing 100 µg/ml ampicillin, and incubate at 37 °C overnight.
6. Analysis of the clones:
    - Seed 3 ml of LB-ampicillin medium in 15 ml sterile tubes with single colonies and incubate the cultures overnight at 37 °C
    - Isolate plasmid DNA using a miniprep plasmid DNA isolation kit
    - Analyze the structure of the plasmids by restriction digestion. The sought-after plasmid (pdCas9-NED) should contain a PacI site. For example, a PacI + EcoRV double digestion of pdCas9-NED should produce 5251 and 2402 bp fragments. Select 2 – 3 plasmids with correct-looking restriction pattern, and verify the presence of the inserted oligonucleotide by sequencing across the AscI and PacI sites. Use sequencing primer 1 or 2 (see 2.1 step 7 A & B).

3.1.2 Construction of dCas9-effector fusions

1. Digestion of pdCas9-NED with AscI and PacI. Prepare the following reaction mixture:

- 2 to 4 µg pdCas9-NED plasmid DNA
- 10 µl 10X FastDigest Green Buffer
- 2 µl FastDigest AscI
- 2 µl FastDigest PacI
- H_2O to 100 µl final volume

Incubate at 37 °C for 1 hour. If the digestion is complete (see 3.1.1. step 1), gel-purify the large (7642 bp) fragment and determine DNA concentration (*see* **Note 5**).

2. PCR-amplification of the effector domain (*see* **Note 6**). The forward primer should contain the 5'-extension AGGCGCGCC consisting of the AscI recognition site (underlined) and an A preceding the AscI site. The function of the 5'-A is to ensure efficient cleavage of the AscI site located at the end of the PCR product. The last three nucleotides of the AscI site (GCC) coincide with a codon (Ala) in the reading frame of the dCas9-NLS-3xFLAG-linker peptide fusion protein, thus the first codon of the effector protein can be placed directly after the AscI site. The reverse primer should contain the 5'-extension GTTAATTAA consisting of the PacI recognition site (underlined) and an extra G at the 5'-end. The function of the 5'-G is to ensure efficient cleavage of the PacI site located at the end of the PCR product. The reverse primer should be designed to contain the complement of an in-frame stop codon. Alternatively, one of the two UAA stop codons offered by the PacI site in different reading frames can be used to terminate translation at the end of the effector domain (*see* **Note 7**). The Pfu PCR reaction mixture (25 µl) contains:

- < 250 ng template DNA

- 1 µl forward primer (20 µM)
- 1 µl reverse primer (20 µM)
- 0.5 µl dNTP (10 µM)
- 10X Pfu buffer with 2.5 µl $MgSO_4$
- 1 µl Pfu DNA polymerase
- H_2O to 25 µl final volume

After establishing the optimal melting temperature to allow the primers to anneal efficiently and specifically, the PCR can be run in a thermocycler. After the PCR reaction, check the size of the amplified fragment by running a 3 µl sample of the reaction in a 1% agarose gel. If the reaction produced a DNA fragment of the correct size, purify the PCR product using a PCR purification kit and measure the concentration of the eluted DNA.

3. Digestion of the PCR product with AscI and PacI:

- 2 to 4 µg PCR product.
- 10 µl 10X FastDigest Green Buffer
- 2 µl FastDigest AscI
- 2 µl FastDigest PacI
- H_2O to 100 µl final volume

Incubate at 37 °C for 2 hours.

Inactivate the restriction enzymes by placing the tubes to 80 °C for 20 min.

4. Cloning the AscI- and PacI-digested PCR product in pdCas9-NED:

- ~200 ng AscI- and PacI-digested pdCas9-NED plasmid
- 200-500 ng AscI- and PacI-digested PCR product
- 2 µl 10x T4 DNA ligase buffer
- 1 µl T4 DNA ligase

- H_2O to 20 µl final volume

Incubate for 30 minutes - 2 hours at room temperature or at 10 – 15 °C overnight (see 3.1.1. step 4). Introduce the ligated DNA into *E. coli* cells by transformation (see 3.1.1. step 5).

5. Isolate plasmid DNA from individual clones using a plasmid miniprep DNA isolation kit and analyze the structure of the plasmids by restriction digestion. Pick 2 – 3 plasmids, which appear to have the correct structure and determine the nucleotide sequence of the inserted effector gene. Use sequencing primer 1 and/or 2 (see 2.1 step 7 A & B).

3.2 Cloning the single guide RNA specifying the addressed genomic sequence

Plasmid MLM3636 contains a sgRNA expression cassette designed to express custom-made sgRNA from a U6 promoter. To obtain a plasmid for guiding the dCas9-effector of interest to a specific genomic site, an oligonucleotide containing the 20 bp target sequence is cloned between the two BsmBI sites of MLM3636 (Figure 2).

1. Determine the target region to which the dCas9-effector fusion protein should bind. Paste the sequence of the wider target region (for example the DNA sequence spanning the – 300 bp to + 150 bp segment surrounding the transcription start side) into an sgRNA design program (e.g. http://crispr.mit.edu/), and pick the best scoring ~20 bp guide sequences.

2. Design the oligonucleotides to be cloned in MLM3636 using the online tool "CRISPR/Cas: Design oligos for making guide RNAs" at http://zifit.partners.org/ZiFiT/ChoiceMenu.aspx. The program will generate a pair of complementary oligonucleotides containing the 20 bp guide (spacer) sequence with

added flanking 5'-C and a 3'-G. The duplex formed by the two oligonucleotides will carry single-stranded overhangs (5'-ACAC and 5'-AAAA) that are complementary to the single-stranded ends generated by BsmBI digestion of MLM3636 (Figure 2). Because efficient transcription from the U6 promoter requires a G as starting base, select target sites, which start with a G in the top strand. The top strand oligonucleotide will have the sequence 5'-ACACCGN_{19}G (Figure 2). Order synthesis of the oligonucleotides.

3. Dissolve the oligonucleotides to 100 µM in TE buffer. Combine:

- 5µl Oligo 1
- 5 µl Oligo 2
- 1 µl 1 M Tris-HCl pH7.5
- 2 µl 1 M NaCl
- 7 µl H_2O

Anneal the oligonucleotides (see 3.1.1. step 2).

4. Digest MLM3636 plasmid DNA with BsmBI in 100 µl digestion mixture:

- 2 to 4 µg MLM3636 plasmid DNA
- 10 µl 10X NEBuffer 3.1
- 2 µl BsmBI
- H_2O to 100 µl final volume

Incubate at 55 °C for 1 hour.

5. Check the completeness of the digestion (see 3.1.1. step 1). If the digestion is complete, purify the linear DNA fragment from a preparative agarose gel (see 3.1.1. step 3). Alternatively, heat-inactivate BsmBI (80 °C, 20 min), and use the digested vector directly for ligation.

6. Ligate the annealed spacer duplex (Oligo top strand + Oligo bottom strand) into the BsmBI-digested MLM3636 plasmid:

- ~200 ng BsmBI-digested MLM3636
- Annealed oligo top strand + oligo bottom strand (2 to 10-fold molar excess over the vector)
- 2µl 10xT4 DNA ligase buffer
- 1 µl T4 DNA ligase
- H_2O to 20 µl final volume

Incubate for 30 minutes - 2 hours at room temperature or at 10 – 15 °C overnight (see 3.1.1. step 4).

6. Introduce the ligated DNA into *E. coli* cells by transformation, select ampicillin-resistant clones (see 3.1.1. step 5).

7. Isolate plasmid DNA from individual clones using a miniprep DNA isolation kit and verify the nucleotide sequence of the inserted guide RNA gene in psgRNA# (Figure 2). Use sequencing primer 3 (see 2.1. step 7 C).

8. Validate the effect of targeted epigenetic editing in transient transfection experiments, using appropriate read-outs (Figure 3).

3.3 Construction of plasmids for stable expression of dCas9-effector fusions

3.3.1 Construction of the vector plasmid pHAGE EF1α dCas9-NED

The lentiviral expression plasmid vector pHAGE EF1α dCas9-VP64 encodes the fusion protein dCas9-VP64 (Figure 4). Transcription of the fusion gene starts from the EF1α promoter. To generate an acceptor plasmid for making dCas9-effector fusions, the VP64 gene is deleted from pHAGE EF1α dCas9-VP64, and replaced with an oligonucleotide containing a MluI (A/CGCGT) and an AsiSI (GCGAT/CGC) site. The new plasmid (pHAGE EF1α dCas9-NED)

(Figure 4) will serve as a vector backbone for cloning effector genes. The effector gene, which has been tested in the transient expression system, is excised from the pdCas9-effector plasmid (see above) by AscI – PacI double-digestion, and transferred into pHAGE EF1α dCas9-NED digested with MluI and AsiSI. Because the single-stranded ends generated by AscI are compatible with MluI ends, and PacI ends are compatible with AsiSI ends, the effector fragment can be transferred directly, in the correct orientation, into pHAGE EF1α dCas9-NED to yield pHAGE EF1α dCas9-ED (Figure 4). The dCas9 and the effector gene will be fused in the correct reading frame.

1. Digestion of pHAGE EF1α dCas9-VP64 with BamHI and NotI. Prepare the reaction mixture:

- 2 to 4 µg pHAGE EF1α dCas9-VP64 plasmid
- 10 µl 10X FastDigest Green buffer
- 2 µl FastDigest BamHI
- 2 µl FastDigest NotI
- H_2O to 100 µl

Incubate at 37 °C for 1 hour. Check the completeness of the digestion (see 3.1.1 step 1). If the digestion is complete, purify the large, 12534 bp DNA fragment from a preparative agarose gel (see 3.1.1. step 3). Measure the concentration of the purified DNA fragment (*see* **Note 8**).

2. Annealing of Oligo 3 and Oligo 4. The duplex will contain an MluI and an AsiSI site and will carry BamHI- and NotI-compatible single-stranded ends.

Dissolve the oligonucleotides to 100 µM in TE buffer. Combine:

- 5µl Oligo 3
- 5 µl Oligo 4

- 1 µl 1 M Tris-HCl pH7.5
- 2 µl 1 M NaCl
- 7 µl H_2O

Anneal the oligonucleotides (see 3.1.1. step 2).

3. Ligation of the annealed oligonucleotides to the purified vector fragment. Prepare the following reaction mixture:

- ~200 ng of the 12 kb fragment of BamHI- and NotI-digested pHAGE EF1α dCas9-VP64
- Annealed Oligos 3+4 (2 to 10-fold molar excess over the vector)
- 2 µl 10x T4 DNA ligase buffer
- 1 µl T4 DNA ligase
- H_2O to 20 µl final volume

Mix gently and incubate for 30 minutes - 2 hours at room temperature or at 10 – 15 °C overnight (see 3.1.1. step 4).

4. Analysis of the clones:

Transform *E. coli* with the ligated DNA. Prepare plasmid DNA from ampicillin-resistant transformants (see 3.1.1. step 5).

The sought-after plasmid pHAGE EF1α dCas9-NED will contain closely located MluI and AsiSI sites. Verify the presence of the inserted oligonucleotide by restriction enzyme analysis using these enzymes individually and/or sequence across the BamHI and NotI sites. Use sequencing primer 4 (see 2.1 step 7 D).

3.3.2 Construction of the plasmids for stable expression of dCas9-effector fusions

1. Isolation of the effector gene from the pdCas9-effector plasmid. Prepare the following reaction mixture:

- 2 to 4 µg pdCas9-effector plasmid
- 10 µl 10X FastDigest Green buffer
- 2 µl FastDigest AscI
- 2 µl FastDigest PacI
- H_2O to 100 µl

Incubate at 37 °C for 1 hour. Check the completeness of the digestion. If the digestion is complete, purify the DNA fragment encoding the effector domain from a preparative agarose gel (see 3.1.1 step 3). Measure the concentration of the purified DNA fragment.

2. Isolation of the pHAGE EF1α dCas9-NED backbone. Prepare the following reaction mixture:
   - 2 to 4 µg pHAGE EF1α dCas9-NED plasmid
   - 10 µl 10X FastDigest Green buffer
   - 2 µl FastDigest MluI
   - 2 µl FastDigest AsiSI
   - H_2O to 100 µl

   Incubate at 37 °C for 1 hour. Check the completeness of the digestion. If the digestion is complete, purify the linear vector fragment from a preparative agarose gel (see 3.1.1. step 3). Measure the concentration of the purified DNA fragment.
3. Cloning of the effector gene in pHAGE EF1α-dCas9-NED. Prepare the following reaction mixture:
   - ~200 ng MluI- and AsiSI-digested pHAGE EF1α dCas9-NED
   - ~200 ng AscI- and PacI-digested effector fragment
   - 2 µl 10x T4 DNA ligase buffer
   - 1 µl T4 DNA ligase

- H_2O to 20 µl final volume

Mix gently and incubate for 30 minutes - 2 hours at room temperature or at 10 – 15 °C overnight (see 3.1.1. step 4).

4. Analysis of the clones:

   Transform *E. coli* with the ligated DNA. Prepare plasmid DNA from ampicillin-resistant transformants (see 3.1.1. step 5). Analyze the structure of the plasmids by restriction digestions.

3.4 Establishing kinetics of epigenetic editing

3.4.1 Construction of stable dCas9-effector expressing cell lines (Figure 5)

1. **Day 0:** Seed the HEK293T cells at a density of 4 x 10^6 cells per 10 cm plate (1 per construct) in 10 ml medium (*see* **Notes 9** and **10**).
2. **Day 1:** TRANSFECTION (*see* **Note 11**) - prepare in a tube the following transfection mixture (per plate/construct):
   - 3.75 ml DMEM serum free
   - 2 µg psPAX2
   - 5 µg pMD2.G-VSV-G
   - 5 µg pHAGE EF1α dCas9-ED
   - 12 µl PLUS reagent

   Mix the content of the tube and incubate at RT for 3-5 minutes.

3. Add 22 µl LTX reagent to the same tube, mix and incubate 20 min at RT.
4. In the meantime, add fresh pre-warmed medium (10 ml) to HEK293T cells plated on day 0 (*see* **Note 12**).
5. After the incubation, pipet the transfection mix carefully (drop by drop) on the medium of the HEK293T cells.

6. Carefully swirl the plate, so that the transfection mix is properly divided.
7. Incubate the plates for 24 hours at 37 °C, in a 5% CO_2 containing humidified incubator
8. **Day 2:** (T=24h after transfection) (*see* **Note 13**) replace the medium of the transfected HEK293T cells with 7 ml fresh medium and incubate for another 24 hours (*see* **Notes 14-16**).
9. Seed your host cells. The density and size of the culture flask/wells depends on the type of experiment you want to perform (*see* **Notes 17** and **18**).
10. **Day 3:** (T=48h after transfection) Transfer the medium of the transfected HEK293T plate (= Virus supernatant) into a 10 ml syringe and filter it through a SFCA 0.40 µ filter into a 15 ml tube, to get rid of the HEK293T cells (*see* **Note 19**), and clean the filter and the syringe by rinsing with disinfectant.
11. Add 7 ml fresh medium to the HEK293T cells. Harvest virus-containing supernatant again at day 4 in the afternoon.
12. TRANSDUCTION of host cells: thaw frozen virus supernatant at 37 ˚C for 20 min or use fresh virus supernatant. As a guideline, use 1.5 ml for 6 wells, 3.5 ml for T25 and 8 ml for T75 culture flasks. Per ml virus supernatant add: 100 µl FBS and 8µg/ml Polybrene Mix to host cells, do NOT add any other medium.
13. **Day 4:** repeat step 10. Store the virus-containing supernatant at -20˚C or add again to the host cells (step 12) (*see* **Note 20**).
14. Add disinfectant solution to the HEK293T plates, let it stand for 15 minutes, aspirate and discard appropriately.
15. After 6-8 hours of the second transduction step of the host cells: aspirate the media of host cells and put them on normal medium.

16. **Day 7:** Aspirate the medium from the host cells and add selection medium (DMEM + 8 µg/ml puromycin) for 4 days (cells should be passaged when confluent).
17. Subsequently the resulting stable cell lines should be cultured in 1 µg/ml puromycin-supplemented medium and can be stored in liquid N_2
18. Upon thawing and subculturing, select for stable expressing cells using 1 µg/ml puromycin-supplemented medium after they have been in culture for 1 week.

3.4.2 Transfection of stable cell lines (*see* Note 21).

The above constructed stable cells will constantly express the dCas9-effector fusions. By transiently transfecting these cells with psgRNA# of interest (Figure 5) (see 3.2.), the long term kinetics of expression modulation can be assessed because the expression of sgRNAs will fade out.

1. **Day 0:** Seed the desired stable cell line with approximately 500,000 cells per well in a six-well plate in 1 µg/ml puromycin-supplemented medium.
2. **Day 1:** Using the desired transfection agent, transfect up to 2µg of the pMLM3636-derived plasmid(s) expressing the sgRNA(s) designed to target your gene of interest.
3. **Day 3:** Collect cells, and quantify RNA expression using standard assays to determine the initial/short-term effects.
4. **Day 12**: Collect cells after a desired number of days (f.e. 12 days) and analyze the long-term kinetics of the epigenetic marks and gene expression using the appropriate read-out assays.

4 Notes

1. Cell lines should always be tested for mycoplasma contamination and can be authenticated using short tandem repeats (STR) profiling.
2. The lab in which viral particles are being handled should at least be of biosafety level 2, allowing work to be carried out with potential pathogenic or infectious organisms posing a moderate hazard. The lab should be equipped with special safety equipment and physical barriers like safety cabinets and biohazard warning signs at all access points. Immunocompromised or immunosuppressed people might be denied access to the lab and immunizations to f.e. hepatitis B might be required. Adhere to local rules regarding application, declaration, and documentation of experiments.
3. Alternatively, the ligation mixture can be incubated at lower temperature (10 – 15 °C) overnight. Prolonged incubation can increase the efficiency of ligation.
4. Because of the close location of the AscI and the AgeI sites, complete digestion of the plasmid is difficult to obtain. Incomplete digestion of one or both sites can result in a large number of empty vectors without inserted oligonucleotide. To estimate the background resulting from such partially digested molecules, setting-up of a "self-ligation" reaction (without the annealed oligos) is recommended.
5. Due to different conditions required by the two enzymes, complete digestion can be difficult to achieve with conventional restriction enzyme preparations. For conventional restriction enzymes, performing the digestion in two steps in optimal buffers with DNA purification in between is recommended, although dilution to change the buffer might work as well.
6. As a control, catalytically inactive mutant effector domains should be used, which can be obtained through site-directed mutagenesis of the wild-type effector.
7. Make sure that the coding sequence of the effector does not contain AscI or PacI site (AscI and PacI recognize 8 bp sites thus cut very rarely).
8. pHAGE EF1α dCas9-VP64 contains two closely located NotI sites. BamHI – NotI double digestion deletes the 64 bp NotI-NotI fragment.
9. Do not let the cells grow to full confluency, 70 % is perfect.
10. Preferably seed in the afternoon of day 0, this ensures a better time-schedule for the rest of the procedure.
11. Preferably in the afternoon (24 h after seeding at a confluency of 70%).
12. HEK293T cells detach very easily. Let the medium slide over the walls of the plate very slowly rather than applying directly on the cells and try to prevent breaking the cell layer.

The more medium added, the less concentrated the virus will be. For direct application to cells this will not be a huge problem, but when freezing the virus supernatant, this could affect infection efficiency. Every freeze-thaw cycle approximately halves the amount of viable virus particles.

13. Should be performed in the afternoon to ensure an optimal time schedule for the rest of the procedure.
14. Be careful not to get contact with bare skin
15. Clean the surfaces and all equipment thoroughly with ethanol.
16. Dispose of virus-containing medium and virus-contaminated pipets by placing these straight into disinfectant in order to inactivate the virus.
17. Should be performed in the afternoon ensure an optimal time schedule for the rest of the procedure.
18. As a guidance: seed 9,000 cells/cm^2 for 3-4 days of recovery after addition of virus.
19. Use directly for infection or store at -20˚C; make sure to label properly: "DANGER active virus" etc.
20. Perform this step early in the morning, so step 15 can be performed in the afternoon.
21. Efficiency of transfection and expression decreases with increasing plasmid size. If possible, do not use plasmids larger than 14 kb.

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Legends to Figures

Figure 1. Construction of plasmids expressing dCas9-effector fusion proteins. The plasmid pMLM3705 encoding a dCas9-VP64 fusion is converted into a general acceptor plasmid pdCas9-NED by digestion with AscI and AgeI. The new plasmid will encode the fusion protein dCas9-SV40 nuclear localization signal (NLS) - 3xFLAG peptide, and will serve as an acceptor for genes of effector domains which can be inserted between the AscI and the PacI sites.

Figure 2. Construction of plasmids expressing single-guide RNA. The double-stranded oligonucleotide specifying the target sequence (20 bp) is cloned between the BsmBI sites of MLM3636 (bold in psgRNS#).

Figure 3. Workflow of transient transfection experiments designed to study the effect of targeting dCas9-linked epigenetic effectors to a genomic site. The plasmids expressing the specific sgRNAs to target the gene of interest (psgRNA) and the pdCas9-ED are transfected in the target cells. After 48 hours, cells are collected and RNA/DNA can be isolated to validate the effect of targeted epigenetic editing , using the appropriate read-outs.

Figure 4. Construction of lentiviral vector plasmids expressing dCas9-effector fusion proteins. The plasmid pHAGE EF1α dCas9-VP64 encoding a dCas9-VP64 fusion is converted into a general acceptor plasmid pHAGE EF1α dCas9-NED by digestion with BamHI and NotI. The new plasmid will containing a MluI (A/CGCGT) and an AsiSI (GCGAT/CGC) site, of which the ends are compatible with AscI and PacI ends, so that effector fragments can be transferred directly, in the correct orientation, to yield pHAGE EF1α dCas9-ED.

Figure 5. Experimental system designed to study the effect of targeting dCas9-linked epigenetic effector to a specific genomic locus in mammalian cell lines permanently expressing the dCas9-effector fusion protein. The plasmid expressing the sgRNA, which determines target specificity, is introduced by transient transfection.